Bisubstrate Kinetic Model for Enzymatic Decolorization of Reactive Black 5 by *Coprinus cinereus* Peroxidase

Mansouri Majoumerd, Mohammad; Kariminia, Hamid Reza*[+●]

Department of Chemical and Petroleum Engineering, Sharif University of Technology, P.O. Box 11155-9465 Tehran, I.R. IRAN

ABSTRACT: *In this study, decolorization of the diazo dye, Reactive Black 5 (RB5) in a Coprinus cinereus peroxidase-catalyzed reaction has been investigated. A bisubstate kinetic model for the reaction rate based on the Ping-Pong mechanism was assumed for the enzymatic decolorization. Experiments were conducted at different RB5 and hydrogen peroxide concentrations in a batch manner to estimate the intrinsic kinetic parameters. These parameters were used for the modeling of decolorization in a continuous reactor that was compared with experimental results. An acceptable agreement was observed between the model and experimental data.*

KEY WORDS: *Coprinus cinereus peroxidase, Decolorization, Reactive black 5, Enzymatic reaction, Ping-Pong mechanism.*

INTRODUCTION

Dyes are extensively used in different industrial processes. Annually, over 7×10^5 tons of dye-stuff are produced worldwide [1,2]. Approximately, about 10–15% of the total dye used in the dyeing process may be released to the waste waters [3,4]. Low concentration of dyes (more than 1 mg/L) in effluents is highly visible and undesirable [5]. Many dyes have been found difficult to decolorize due to their complex structure and considered to be recalcitrant. They resist microbial biodegradation and are therefore, not easily degraded in waste water treatment plants [6]. Dyes have many structural varieties, such as, acidic, basic, disperse, azo, diazo, anthroquinone based and metal complex dyes [7]. Azo dyes are the most commonly used dyes in textile, food, paper and cosmetic industries. Waste waters from these industries are highly colored wherein the residual azo reactive dyes are generally resistant to microbial degradation. Therefore, treatment of synthetic dyes in waste water is a matter of great concern. Several physico-chemical methods have been employed for the removal of dyes [8]. However, these procedures have not been widely used due to their high cost, intensive energy demand and formation of hazardous by products [9]. Moreover, dye effluents are poorly decolorized by conventional biological treatment methods and may be toxic for the microorganisms present in such treatment plants [8].

Recently, researchers have been focusing their attention towards developing processes in which enzymes

* *To whom correspondence should be addressed.*

+ *E-mail: kariminia@sharif.ir*

● *Other Address: Institute for Biotechnology and Environment, Sharif University of Technology, P.O. Box 11155-1639 Tehran, I.R. IRAN*

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are employed to remove dyes from polluted water [10-12]. The potential advantages of enzymatic treatment as compared to other biological treatments are mainly associated with several factors such as possible operation at high or low concentrations of dye, shorter treatment period, absence of lag phase compared to microbial treatment, sludge volume reduction and ease of controlling the process [13,14]. Peroxidases (EC 1.11.1.7) are versatile biocatalysts with wide spectrum applications in a number of industrial processes and have already been employed for the transformation of toxic compounds of industrial origin such as aromatic compounds to preserve the quality of water [11,15,16]. Many peroxidases such as lignin peroxidase, manganese peroxidase, soybean peroxidase, HorseRadish Peroxidase (HRP) and laccase, have been applied for enzymatic treatment to decolorize and degrade dye in industrial effluents [13, 17, 18]. There are also several reports on the enzymatic oxidation of phenolic compounds [19-21].

Limited number of studies has been focused on the kinetics of peroxidase catalyzed reactions using Michaelis-Menten kinetic mechanism. *Tong et al.* have described that the oxidation reaction system of phenol, 4-chlorophenol and 3-chlorophenol catalyzed by horseradish peroxidase in the presence of hydrogen peroxide, follows the bisubstrate ping-pong mechanism. They also calculated the kinetic parameters of different hydrogen peroxide concentrations, demonstrating that an optimum hydrogen peroxide concentration exists, above which hydrogen peroxide becomes a reaction inhibitor [22]. *Wu et al.* developed a kinetic model for the phenol/horseradish peroxidase system in the presence of polyethylene glycol. The phenol oxidation was described using a Michaelis–Menten bisubstrate equation [23]. *Wright & Nicell*, developed a model showing the dependence of the enzyme activity on the hydrogen peroxide concentration and calculated the kinetic parameters, which were lower than those obtained for HRP [21]. *Liu et al.* proposed the Ping-Pong bisubstrate kinetics mechanism for a system containing hydrogen peroxide, *o*-phenylenediamine and horseradish peroxidase. B´odalo et al. assumed the Ping-Pong mechanism for the removal of 4-chlorophenol with soybean peroxidase/hydrogen peroxide and used a set of experimental data in a batch reactor to estimate kinetic parameters [24]. *G´omez et al.* studied the removal of 4-chlorophenol from aqueous solutions using soybean peroxidase assuming a Ping-Pong bisubstrate kinetic for the enzymatic reaction [25]. *Nazari et al.* focused on the inhibition rule of suicide-substrate of microperoxidase-11 in oxidation reaction of guaiacol by hydrogen peroxide in the presence of amino acids. They used unisubstrate simple Michaelis- Menten mechanism with an acceptable fitness [26]. There are limited number of studies on the kinetics of enzymatic reaction for decolorization [27,28].

We had previously studied dye decolorization by *Coprinus Cinereus* peroxidase in both batch [29] and continuous [30] systems and investigated the optimum conditions. Here, we have focused on the kinetics of dye decolorization by *Coprinus Cinereus* peroxidase. In this research, similar mechanism proposed for oxidation of phenolic compounds based on the bisubstarte Ping-Pong mechanism has been utilized to estimate the reaction kinetics for enzymatic oxidation of dye. Parameters have been derived from experimental data in the batch mode and used to predict the behavior of continuous decolorization. The main objective of this work was to determine kinetic parameters for enzymatic decolorization of RB5 by *Coprinus Cinereus* Peroxidase (CIP) and predict the decolorization efficiency in the continuous mode.

THEORITICAL SECTION

Mechanism of peroxidase catalyzed reaction

G´omez et al. have described the mechanism of phenol degradation in the CIP catalized reaction [31]. As shown in Fig. 2, peroxidase is firstly oxidized by hydrogen peroxide to a catalytically active form called Compound I (E_1), which can oxidize a phenolic substrate into a phenoxy radical through oxidation of one-electron. Another phenoxy radical will be generated from the oxidation of a second phenolic substrate by Compound II (E_2) (the reduced form of E_1) catalysis, and the enzyme will be returned to its resting state. In the presence of excess amount of hydrogen peroxide, E_2 can be oxidized to Compound III (E_3), which appears as an inactive form of the enzyme. E_3 is either decomposed to the resting enzyme or returns to E_1 form through another phenol oxidation stage [32].

Usually, oxidation of aromatic compounds catalyzed by peroxidase, has been described through the mechanism known as the Dunford mechanism [20].

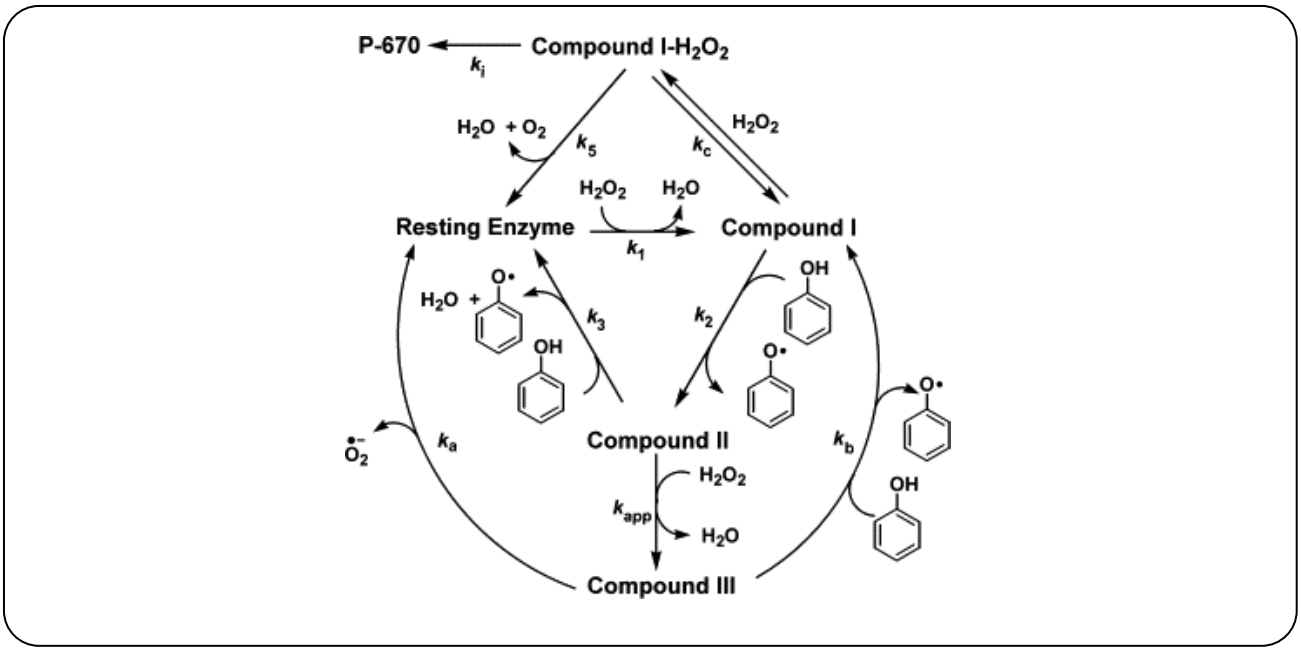

Fig. 1: Catalytic cycle of peroxidase catalyzed phenol degradation. P-670 is a permanently inactivated form of enzyme [32].

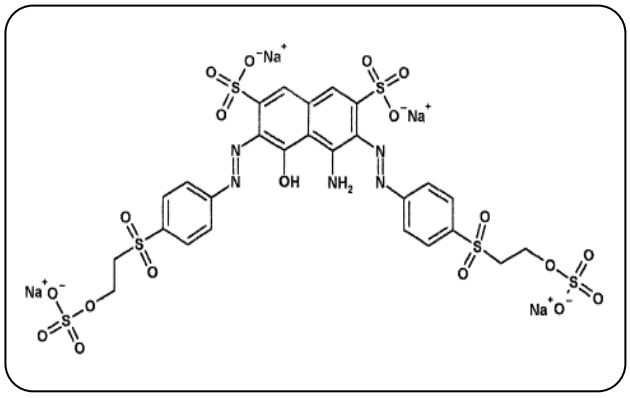

Fig. 2: Chemical structure of Reactive lack 5 [37].

For ease of understanding, direct kinetics mechanism that is a generally accepted for the peroxidase-catalyzed reaction is presented here [33]:

$$\text{Enzyme} + H_2O_2 \rightarrow E_1 \quad (1)$$

$$E_1 + \Phi H_2 \rightarrow E_2 + \Phi H^{\bullet} \quad (2)$$

$$E_2 + \Phi H_2 \rightarrow \text{Enzyme} + \Phi H^{\bullet} \quad (3)$$

$$AH^{\bullet} + \Phi H^{\bullet} \rightarrow H\Phi - \Phi H \quad (4)$$

In this simplest form of kinetics mechanism, ΦH_2 and $\Phi H^{\bullet}$ are aromatic compound and free radical, respectively. After formation of free radicals in the oxidation of compound I and compound II, free radicals will bond to each other and close the catalytic cycle.

The overall reaction is:

$$H_2O_2 + 2\Phi H_2 \xrightarrow{\text{(enzyme)}} 2\Phi H^{\bullet} + 2H_2O \quad (5)$$

Excess amount of hydrogen peroxide can oxidize E_2 to E_3, which appears as an inactive form of the enzyme:

$$E_2 + H_2O_2 \rightarrow E_3 + H_2O \quad (6)$$

This is not an irreversible deactivation because E_3 breaks down spontaneously to the native form although with a low reaction rate [31]:

$$E_3 \rightarrow \text{Enzyme} + O_2^{-} \quad (7)$$

A group of researchers have focused on the oxidation of substituted phenolic compounds by the HRP Compound II. According to their study, HRP has an oxidation state above that of the native enzyme, which is a hemo-protein with a Fe^{III} nucleus. Regarding to the reaction products, dimeric compounds can appear in the bulk reaction as a result of the binding of two radicals formed from the aromatic compound. Although the resulting dimer is less soluble, it remains in the aqueous solution where it can be oxidized again in successive steps, leading to the formation of a longer chain polymer whose precipitation is favored. This requires additional

consumption of hydrogen peroxide and influences the stoichiometry of the overall process, which moves toward gradually higher hydrogen peroxide/phenol molar ratios, depending on the degree of polymerization achieved [34].

According to the Dunford mechanism and the deactivation phenomena, there are three kinetic models for the HRP/hydrogen peroxide/4-chlorophenol system, in steady-state, fully transient and pseudo-steady-state conditions, respectively. As *Nicell* pointed out, the steady-state model leads to an equation similar to the Ping Pong bisubstrate enzymatic kinetic model [35]. The transient-state model has been formulated as a series of nine simultaneous differential equations, containing ten kinetic parameters to be determined by fitting method. Due to the model complexity, it must be solved numerically that requires long computing times. Despite this drawback, the model has provided a good fitting with the experimental data. In the pseudo-steady-state model, a steady-state approximation is assumed with some intermediate reactions. Despite certain simplifications, the model still remains complicated and must be solved by numerical method [36]. The fitting of the model with the experimental data is 2% less precise than that for the fully transient model. This loss of precision is valued in turn of simplicity and reduced computation time.

Assumptions for peroxidase catalyzed reaction

According to the original Dunford mechanism, following issues were taken into account, in our work:

- The reaction takes place under moderate values of the hydrogen peroxide/dye ratio, and the formation of Compound III is negligible.
- The overall process consists of an undetermined number of catalytic cycles that depends on the operational conditions.

In this work, the commonly accepted bisusbtrate Ping-Pong equation for the initial reaction rate, r_0 was adopted as Eq. (8).

$$r_0 = \frac{K_{cat}.[E]_0.[C]_0.[H]_0}{K_M^{H_2O_2}.[C]_0 + K_M^{Dye}.[H]_0 + [C]_0.[H]_0} \quad (8)$$

Estimation of kinetic parameters (in batch mode experiments)

Series of experiments were carried out at different initial dye concentrations. Eq. (8) was rearranged to the form given in Eq. (9) which provides groups of straight lines with ordinates on the origin as a linear function of the inverse of the hydrogen peroxide concentration.

$$\frac{1}{r_0} = \left(\frac{1}{V_{Max}} + \frac{K_M^{H_2O_2}}{V_{Max}} \cdot \frac{1}{[H]_0}\right) + \frac{K_M^{Dye}}{V_{Max}} \cdot \frac{1}{[C]_0} \quad (9)$$

Differentiation of Eq. (9) with respect to the initial dye concentration, gives:

$$\frac{d}{d[C]_0}\left(\frac{1}{r_0}\right) = -\frac{K_M^{Dye}}{V_{Max}} \cdot \frac{1}{[C]_0^2} \quad (10)$$

Eq. (10) has the limitation of representation since large variations in the inverse of the initial rate occurs in low concentrations of dye. However, in this condition, dye becomes the limiting reagent. Given a high initial reaction rate, the dye is consumed within the first few moments, hindering correct measurement of r_0. For the same reason, when the dye concentration is much higher than that of hydrogen peroxide, prediction of r_0 value is not reliable. In order to tackle these problems, *B´odalo et al.* proposed another form of linear representation (Eq. (11)), which has proved to be less sensitive to errors in measuring the initial reaction rate [24]:

$$\frac{[C]_0.[H]_0}{r_0} = \frac{K_M^{Dye}}{V_{Max}} \cdot [H]_0 + \left(\frac{K_M^{H_2O_2}}{V_{Max}} + \frac{1}{V_{Max}} \cdot [H]_0\right) \quad (11)$$

In the next step, series of experiments were carried out at different hydrogen peroxide concentrations. Eq. (8) was rearranged to Eq. (12) which resulted in straight lines with ordinate on the origin that is linearly correlated with the inverse of the dye concentration.

$$\frac{1}{r_0} = \left(\frac{1}{V_{Max}} + \frac{K_M^{Dye}}{V_{Max}} \cdot \frac{1}{[C]_0}\right) + \frac{K_M^{H_2O_2}}{V_{Max}} \cdot \frac{1}{[H]_0} \quad (12)$$

For similar reasons that were considered for Eq. (10), a rearrangement was proposed by *B´odalo et al.* in the form of equation (13) [24]:

$$\frac{[C]_0.[H]_0}{r_0} = \frac{K_M^{H_2O_2}}{V_{Max}} \cdot [C]_0 + \left(\frac{K_M^{Dye}}{V_{Max}} + \frac{1}{V_{Max}} \cdot [C]_0\right) \cdot [H]_0 \quad (13)$$

Eqs. (11) and (13) were used to estimate the values for the parameters of $K_M^{H_2O_2}$, K_M^{Dye} and V_{Max}.

Modeling of continuous enzymatic decolorization

The kinetic parameters obtained in the batch experiments were applied to the continuous system. A model for a continuous tank reactor was derived based on the following assumptions:

- The reactor is perfectly mixed and isothermal condition is maintained.
- The inlet and outlet flow rates are equal and, as a result, the reactor volume remains constant throughout the operating time.
- The enzymatic reaction follows the bisubstrate Ping-Pong kinetic mechanism.
- Deactivation of enzyme is negligible.
- Reaction products continuously leave the reactor.

A mass balance on both substrates (dye and hydrogen peroxide) in the single adding mode of enzyme, is:

$$\text{Input} - \text{Output} - \text{Reaction} + \text{Production} = \text{Accumulation} \quad (14)$$

By arranging Eq. (14) in terms of mathematical representations, two ordinary differential equations were obtained:

$$F_D[C]_0 - F_D[C] - V_r\left(\frac{V_{Max}.[E].[C].[H]}{K_M^{H_2O_2}.[E] + K_M^{Dye}.[H] + [C].[H]}\right) = V_r\left(\frac{d[C]}{dt}\right) \quad (15)$$

$$F_H[H]_0 - F_H[H] - V_r\left(\frac{V_{Max}.[E].[C].[H]}{K_M^{H_2O_2}.[C] + K_M^{Dye}.[H] + [C].[H]} \cdot y\left(\frac{H_2O_2}{Dye}\right)\right) = V_r\left(\frac{d[H]}{dt}\right) \quad (16)$$

Enzyme concentration in the above equations was replaced with the Eq. (17) obtained from a balance over the enzyme activity.

$$[E] = [E]_0 \cdot e^{-\frac{t}{\tau}} \quad (17)$$

MATLAB 8.1 software was used to solve the resulting differential equations shown in Eq. (18) and Eq. (19), simultaneously.

$$[C]_0 - [C] - \tau\left(\frac{V_{Max} \cdot e^{\left(-\frac{t}{\tau}\right)} \cdot [C] \cdot [H]}{K_M^{H_2O_2} \cdot [C] + K_M^{Dye} \cdot [H] + [C] \cdot [H]}\right) = \tau\left(\frac{d[C]}{dt}\right) \quad (18)$$

$$F_H[H]_0 - F_H[H] - V_r \cdot \left(\frac{V_{Max} \cdot e^{\left(-\frac{t}{\tau}\right)} \cdot [C] \cdot [H]}{K_M^{H_2O_2} \cdot [C] + K_M^{Dye} \cdot [H] + [C] \cdot [H]} \cdot \left(\frac{H_2O_2}{Dye}\right)\right) = V_r\left(\frac{d[H]}{dt}\right) \quad (19)$$

EXPERIMENTAL SECTION

Materials

The Reactive Blue 5 (RB5) dye was a product of Sumitomo Chemical Co., Japan. All other chemicals were of analytical grade that were prepared from available commercial companies.

Fungal strain and culture conditions

The fungal strain was *Coprinus cinereus* NBRC 30628, obtained from National Biological Resources Center, Japan. Stock cultures were maintained on 39 g/L Potato Dextrose Agar (PDA) slants at 4 °C. For peroxidase production, a liquid medium containing (per liter) glucose 30 g; yeast extract 5 g and pepton 10 g, was used. The pH of the medium was adjusted to 4. Liquid media were sterilized at 121 °C for 20 min. Fungi grown on PDA plates (32 °C for 7 days) were utilized for inoculation. One mycelial piece (1 cm diameter) was taken from the preculture and transferred into the sterilized liquid medium. Cultures were incubated on a rotary shaker-incubator (160 rpm) at 32 °C. Samples (0.1 mL of the culture liquid) were taken daily from each Erlenmeyer flask after the day 19 of inoculation. Peroxidase activity reached to its maximum value after 28 days where the cultivation was terminated. The culture was filtered and the clear liquid was used as crude enzyme for decolorization experiments.

Peroxidase activity assay

Coprinus cinereus peroxidase activity was assayed by colorimetric method [38]. Reaction mixture containing

10 mM phenol, 3.1 mM hydrogen peroxide, 0.97 g/L Triton X-100 and 0.63 mM 4-aminoantipyrin (4-AAP) in a total volume of 3.0 mL was incubated at 37 °C. All reagents were dissolved in 65 mM phosphate buffer (pH 7.0). The reaction was then started by adding 0.1 mL of diluted enzyme solution and the initial increase in the absorbance was monitored at 500 nm during 1 min. Reaction was stopped by the addition of 3.1 M of sodium azide. Under such conditions, the rate of formation of colored product was calculated using the molar extinction coefficient of 12.6 1/M.cm. One unit of peroxidase activity was defined as the amount of the enzyme consuming 1 mol of hydrogen peroxide per minute under the assay conditions.

Dye solution and quantification

RB5 was used in decolorization experiments by CIP. The chemical formula of this dye is $C_{26}H_{21}N_5O_{19}S_6Na_4$ with the molecular weight of 991.8 g/mole and water solubility of 82 g/L at 20 °C [39]. Structure of this dye is represented in Fig. 2. Dye solutions of RB5 (20 to 80 mg/L) were prepared in 0.1 M phosphate buffer solution (pH 8).

Colorimetric method was used for quantitative estimation of dye concentration. Absorbance of the dye solutions was determined at the maximum wavelength of absorbance (596 nm) for the dye. Decolorization Efficiency (DE) was defined as follows:

$$DE(\%) = \left(\frac{\text{Concentration of in itial dye solution}}{\text{concentration of initial dye solution}} - \frac{\text{concentration of decolorized dye solution}}{\text{concentration of initial dye solution}} \right) \times 100 \quad (20)$$

Decolorization in batch mode

Enzymatic decolorization was examined in a batch mode using CIP at pH 8.0 (0.1 M sodium phosphate buffer), in the presence of variable concentrations of hydrogen peroxide at room temperature under shaking condition. Spectrophotometrical method was utilized to measure the residual dye concentration.

Two series of experiments were carried out: (I) effect of dye concentration (20, 40, 60 and 80 mg/L) was investigated, using a constant hydrogen peroxide concentration of 0.25 mM. (II) effect of hydrogen peroxide concentration (0.125, 0.25 and 0.26 mM) was investigated, using a constant dye concentration of 40 mg/L.

Decolorization in continuous mode

Enzymatic dye decolorization by CIP (5 U/mL) was conducted in a 50 mL reactor under stirring condition at room temperature. A constant dye solution flow rate (0.83 mL/min) was provided by a syringe pump. The influent dye concentration was between 20 to 80 mg/L. As much as 250 U of CIP was initially poured inside the reactor. Hydrogen peroxide solution (4.1 mg/L) was introduced into the rector with the flowrate of 0.04 mL/min.

RESULTS AND DISCUSSION

The model, employed in this work consisted of the following parameters: $K_M^{H_2O_2}$, K_M^{Dye} and V_{Max}. Eq. (8) representing the initial reaction rate according to Ping-Pong mechanism was used to estimate these parameters.

Ping-Pong equation and intrinsic parameter

Estimation of initial reaction rate by extrapolation of measured dye concentrations to the time zero, is not a reliable method. Therefore, we used the method proposed by *G´omez et al.* [25] for determination of kinetic parameters.

Different initial dye concentration (batch mode experiments)

Experiments were carried out at different initial dye concentrations (20, 40, 60 and 80 mg/L). Based on Eq. (11), $\frac{[C]_0 \cdot [H]_0}{r_0}$ was sketched against dye concentration as indicated in Fig. 3:

$$\frac{[C]_0 \cdot [H]_0}{r_0} = 0.265 \cdot [C]_0 + 0.459 \ , \ R^2 = 0.962 \quad (21)$$

From the ordinate on the origin, following equations were obtained:

$$\frac{K_M^{Dye}}{V_{Max}} = 0.054 \quad (22)$$

$$\left(\frac{K_M^{H_2O_2}}{V_{Max}} + \frac{1}{V_{Max}} \cdot [H]_0 \right) = 0.265 \quad (23)$$

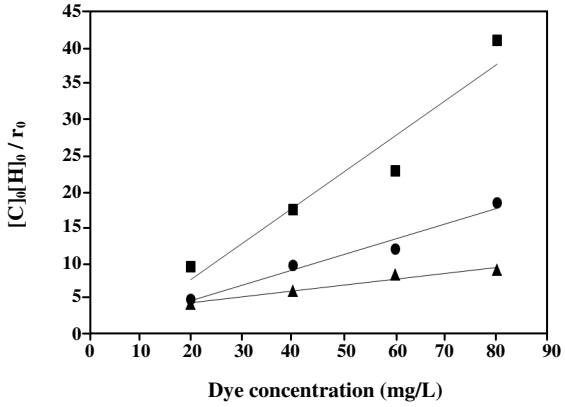

Fig. 3: Series of experiments (▲: 0.5; ●: 1; ■: 2 min) for different RB5 concentrations and a constant hydrogen peroxide concentration of 0.25 mM; based on Eq. (11).

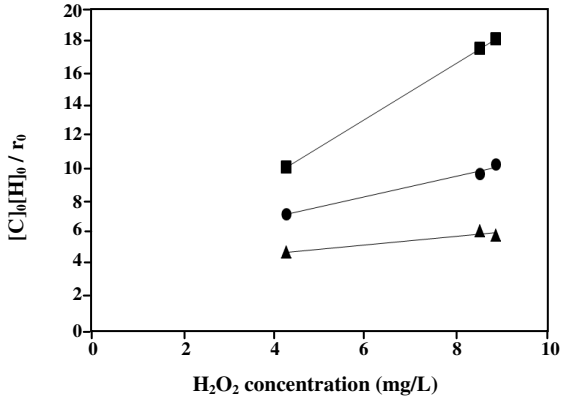

Fig. 4: Series of experiments (▲: 0.5; ●: 1; ■: 2 min) for different hydrogen peroxide concentrations and a constant RB5 concentration of 40 mg/L; based on Eq. (13).

Different hydrogen peroxide concentration (batch mode experiments)

Experiments were carried at different concentrations of hydrogen peroxide (0.125, 0.25 and 0.26 mM). In accordance with Eq.(13), $\frac{[C]_0 \cdot [H]_0}{r_0}$ versus hydrogen peroxide concentration was sketched (Fig. 4):

$$\frac{[C]_0 \cdot [H]_0}{r_0} = 0.879 \cdot [H]_0 + 3.684 \quad , \quad R^2 = 0.975 \qquad (24)$$

From the ordinate on the origin, following equations were obtained:

$$\frac{K_M^{H_2O_2}}{V_{Max}} = 0.092 \qquad (25)$$

$$\left(\frac{K_M^{Dye}}{V_{Max}} + \frac{1}{V_{Max}} \cdot [C]_0 \right) = 0.879 \qquad (25)$$

Intrinsic values of the Michaelis-Menten constants, using Eq. (22) to (26) were calculated as follows:

$K_M^{H_2O_2} = 41.83 \text{ mg/L}$, $K_M^{Dye} = 24.55 \text{ mg/L}$ and, $V_{Max} = 454.63 \text{ mg/(min.L)}$

Continuous dye decolorization (experiment and modeling)

Experimental results of dye decolorization in the continuous system are indicated in Fig. 5 for different initial dye concentrations (20, 40, 60 and 80 mg/L). Higher decolorization efficiencies were observed at lower initial dye concentrations. Model predictions for decolorization efficiency at different initial dye concentrations are also indicated in the same figure. Model was solved applying the parameters estimated in the batch mode experiments. An agreeable fitness of the model with the experimental data was observed. This consistency confirms the validity of using the dependency of enzyme activity and the corresponding decolorization efficiency to hydrogen peroxide concentration (i.e. the concept of Ping-Pong mechanism). This also confirms the correctness of generalization of the derived intrinsic parameters from the batch experiments to the continuous conditions. Furthermore, the good approximation of the model, confirms the accuracy of general hypotheses which have been used for simplification in the modeling of continuous process.

The maximum dye decolorization efficiency of the model was lower than the experimental data for initial dye concentrations of 20 and 40 mg/L (Fig. 5A and B), while it was higher than the experimental data for initial dye concentrations of 60 and 80 mg/L (Fig. 5C and D). This difference between the model and the experiment might be related to the effect of H_2O_2 as an activator and or inhibitor which has not been considered in the model. This means that, under the moderate values of the H_2O_2 concentration/dye concentration, the formation of Compound III has been neglected.

CONCLUSIONS

A bisubstrate Ping-Pong mechanism was applied for the kinetic study of the *Coprinus cinereus* peroxidase catalyzed decolorization of reactive black 5. The equation

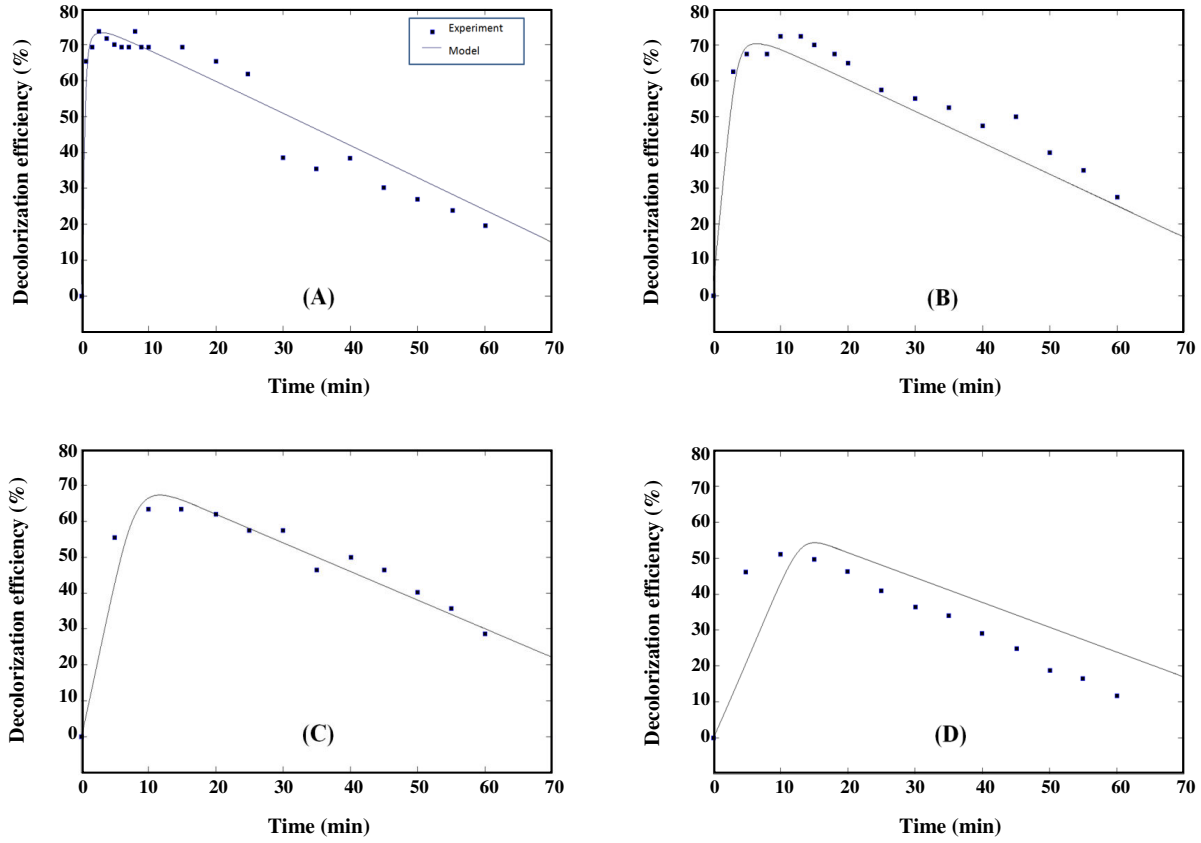

Fig. 5: Comparison between experimental data and model prediction. (A): initial dye concentration, 20 mg/L; (B): initial dye concentration, 40 mg/L; (C): initial dye concentration, 60 mg/L; (D): initial dye concentration, 80 mg/L.

used for the reaction rate was related to Ping-Pong bisubstrate kinetics at time zero. Three sets of experimental date were used for extrapolating to time zero. Series of experiments were conducted in which intrinsic reaction parameters (maximum reaction rate and Michaelis–Menten constants of both substrates) were obtained. These estimated parameters were applied to model the decolorization in a continuous reactor. Experimental results of decolorization were compared to the model prediction and an acceptable agreement was observed which confirmed the validity of the used kinetics and intrinsic parameters.

Nomenclatures

τ	Hydraulic retention time, min
[C]	Concentration of dye, mg/L
$[C]_0$	Initial concentration of dye, mg/L
DE	Decolorization efficiency (%)
[E]	Enzyme concentration, mg/L
$[E]_0$	Total enzyme
F_D	Flow rate of dye, mL/min
F_H	Flow rate of hydrogen peroxide, mL/min
[H]	Concentration of hydrogen peroxide, mg/L
$[H]_0$	Initial concentration of hydrogen peroxide, mg/L
K_{cat}	Enzyme catalytic constant in dye - oxidizing reaction
K_M^{Dye}	Dye Michaelis - Menten constants in the kinetic reaction, mg/L
$K_M^{H_2O_2}$	Hydrogen peroxide michaelis - Menten constants in the kinetic reaction, mg/L
r_0	Initial reaction rate of Dye (mg/(L.min))
$V_{max} = K_{cat}.[E]_0$	maximum reaction rate
V_r	Reactor volume, mL
$y(H_2O_2/Dye)$	Yield ratio of consumption of hydrogen peroxide to dye
$\left(\frac{\partial C_i}{\partial t}\right)$	Time difference of dye concentration, mg/(L.min)

$\left(\frac{\partial H_i}{\partial t}\right)$ Time difference of hydrogen peroxide concentration, mg/(L.min)

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